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Nucleic acids which encode insect acetylcholine receptor subunits

The invention relates, in particular, to nucleic acids which encode insect acetyl-
5 choline receptor subunits.

BACKGROUND OF THE INVENTION

Nicotinic acetylcholine receptors are ligand-regulated ion channels which are of im-
portance in neurotransmission in the animal kingdom. The binding of acetylcholine
or other agonists to the receptor induces a transient opening of the channel and al-
10 lows cations to flow through. It is assumed that a receptor consists of five subunits
which are grouped around a pore. Each of these subunits is a protein which consists
of an extracellular N-terminal moiety followed by three transmembrane regions, an
intracellular moiety, a fourth transmembrane region and a short extracellular C-ter-
minal moiety (Changeux et al. 1992).

15 Acetylcholine receptors are especially well investigated in vertebrates. In this con-
text, three groups can be distinguished on the basis of their anatomical location and
their functional properties (conducting properties of the channel, desensitization, and
sensitivity towards agonists and antagonists and also towards toxins such as
20 α -bungarotoxin). The classification correlates with the molecular composition of the
receptors. There are heterooligomeric receptors having the subunit composition
 $\alpha_2\beta\gamma\delta$, which are found in muscle (Noda et al. 1982, Claudio et al. 1983, Devillers-
Thiery et al. 1983, Noda et al. 1983a, b), heterooligomeric receptors which contain
subunits from the $\alpha 2 - \alpha 6$ and $\beta 2 - \beta 4$ groups and which are found in the nervous
system (Wada et al. 1988, Schoepfer et al. 1990, Cockcroft et al. 1991, Heinemann et
al. 1997), and also homooligomeric receptors which contain subunits from the $\alpha 7 -$
 $\alpha 9$ group and which are likewise found in the nervous system (Lindstrom et al. 1997,
Elgoeyen et al. 1997). This classification is also supported by an examination of the
relatedness of the gene sequences of the different subunits. Typically, the sequences
of functionally homologous subunits from different species are more similar to each
other than are sequences of subunits which are from different groups but from the

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same species. Thus, the rat muscle α subunit, for example, exhibits 78% amino acid identity and 84% amino acid similarity with that of the electric ray *Torpedo californica* but only 48% identity and 59% similarity with the rat $\alpha 2$ subunit (hetero-oligomeric, neuronal) and 36% identity and 45% similarity with the rat $\alpha 7$ subunit (homooligomeric, neuronal). Furthermore, the gene sequences of all the known acetylcholine receptor subunits are to a certain extent similar not only to each other but also to those of some other ligand-regulated ion channels (e.g. the serotonin receptors of the 5HT₃ type, the GABA-regulated chloride channels and the glycine-regulated chloride channels). It is therefore assumed that all these receptors are descended from one common precursor and they are classified into one supergene family (Ortellis et al. 1995).

In insects, acetylcholine is the most important excitatory neurotransmitter of the central nervous system. Accordingly, acetylcholine receptors can be detected electrophysiologically in preparations of insect central nervous system ganglia. The receptors are detected both in postsynaptic and presynaptic nerve endings and in the cell bodies of interneurones, motor neurones and modulatory neurones (Breer et al. 1987, Buckingham et al. 1997). Some of the receptors are inhibited by α -bungarotoxin while others are insensitive (Schloß et al. 1988). In addition, the acetylcholine receptors are the molecular point of attack for important natural (e.g. nicotine) and synthetic insecticides (e.g. chloronicotinyls).

The gene sequences of a number of insect nicotinic acetylcholine receptors are already known. Thus, the sequences of five different subunits have been described in *Drosophila melanogaster* (Bossy et al. 1988, Hermanns-Borgmeyer et al. 1986, Sawruk et al. 1990a, 1990b, Schulz et al. Unpublished, EMBL accession number Y15593), while five have likewise been described in *Locusta migratoria* (Stetzer et al. unpublished, EMBL accession numbers AJ000390 - AJ000393), one has been described in *Schistocerca gregaria* (Marshall et al. 1990), two have been described in *Myzus persicae* (Sgard et al. unpublished, EMBL accession number X81887 and X81888), and one has been described in *Manduca sexta* (Eastham et al. 1997). Fur-

thermore, a number of partial gene sequences from *Drosophila melanogaster* have been characterized as so-called expressed sequence tags (Genbank accession numbers AA540687, AA698155, AA697710, AA697326). The fact that individual sequences are very similar to those from other insects suggests that these subunits are functional
5 homologues.

BRIEF SUMMARY OF THE INVENTION

It is of great practical importance to make available new insect acetylcholine receptor subunits, for example for the purpose of searching for novel insecticides, with those subunits which differ from the known subunits to a greater extent than is the case
10 between functional homologues being particularly of interest.

The present invention is consequently based, in particular, on the object of making available nucleic acids which encode novel insect acetylcholine receptor subunits.

15 This object is achieved by the provision of nucleic acids which comprise a sequence selected from

- (a) the sequences according to SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5,
- 20 (b) part sequences of the sequences defined in (a) which are least 14 base pairs in length,
- (c) sequences which hybridize to the sequences defined in (a) in 2 x SSC at 60°C, preferably in 0.5 x SSC at 60°C, particularly preferably in 0.2 x SSC at 60°C
25 (Sambrook et al. 1989),
- (d) sequences which exhibit at least 70% identity with the sequences defined in (a), between position 1295 and position 2195 in the case of SEQ ID NO: 1, or between position 432 and position 1318 in the case of SEQ ID NO: 3, or be-
30 tween position 154 and position 1123 in the case of SEQ ID NO: 5,

(e) sequences which are complementary to the sequences defined in (a), and
(f) sequences which, because of the degeneracy of the genetic code, encode the same amino acid sequences as the sequences defined in (a) to (d).

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The degree of identity of the nucleic acid sequences is preferably determined using the GAP program from the GCG program package, Version 9.1 with standard settings (Devereux et al. 1984).

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DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the surprising finding that insects possess genes which encode subunits of, in particular, homooligomeric acetylcholine receptors.

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The invention furthermore relates to vectors which contain at least one of the novel nucleic acids. All the plasmids, phasmids, cosmids, YACs or artificial chromosomes which are used in molecular biological laboratories can be used as vectors. These vectors can be linked to the usual regulatory sequences for the purpose of expressing the novel nucleic acids. The choice of such regulatory sequences depends on whether prokaryotic or eukaryotic cells, or cell-free systems, are used for the expression. The SV40, adenovirus or cytomegalovirus early or late promoter, the lac system, the trp system, the main operator and promoter regions of phage lambda, the control regions of the fd coat protein, the 3-phosphoglycerate kinase promoter, the acid phosphatase promoter and the yeast α -mating factor promoter are examples of expression control sequences which are particularly preferred.

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In order to be expressed, the nucleic acids according to the invention can be introduced into suitable host cells. Both prokaryotic cells, preferably E.coli, and eukaryotic cells, preferably mammalian or insect cells, are suitable for use as host cells. Other examples of suitable unicellular host cells are: Pseudomonas, Bacillus, Streptomyces, yeasts, HEK-293, Schneider S2, CHO, COS1 and COS7 cells, plant cells in cell culture and also amphibian cells, in particular oocytes.

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The present invention also relates to polypeptides which are encoded by the nucleic acids according to the invention and also the acetylcholine receptors, preferably homooligomeric acetylcholine receptors, which are synthesized from them.

5 In order to prepare the polypeptides which are encoded by the nucleic acids according to the invention, host cells which contain at least one of the nucleic acids according to the invention can be cultured under suitable conditions. After that, the desired polypeptides can be isolated from the cells or the culture medium in a customary manner.

10 The invention furthermore relates to antibodies which bind specifically to the above-mentioned polypeptides or receptors. These antibodies are prepared in the customary manner. For example, such antibodies can be produced by injecting a substantially immunocompetent host with a quantity of an acetylcholine receptor polypeptide, or a fragment thereof, according to the invention which is effective for producing antibodies, and subsequently isolating these antibodies. Furthermore, an immortalized cell line which produces monoclonal antibodies can be obtained in a manner known per se. Where appropriate, the antibodies can be labelled with a detection reagent. Preferred examples of such a detection reagent are enzymes, radioactively labelled elements, fluorescent chemicals or biotin. Instead of the complete antibody, use can also be made
15 of fragments which possess the desired specific binding properties.
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25 The nucleic acids according to the invention can be used, in particular, for producing transgenic invertebrates. These latter can be employed in test systems which are based on an expression of the receptors according to the invention, or variants thereof, which differs from that of the wild type. In addition, this includes all transgenic invertebrates in which a change in the expression of the receptors according to the invention, or their variants, occurs as the result of modifying other genes or gene control sequences (promoters).

The transgenic invertebrates are produced, for example, in *Drosophila melanogaster* by means of P element-mediated gene transfer (Hay et al., 1997) or in *Caenorhabditis elegans* by means of transposon-mediated gene transfer (e.g. using *Tc1*, Plasterk, 1996).

5 The invention also consequently relates to transgenic invertebrates which contain at least one of the nucleic acid sequences according to the invention, preferably to transgenic invertebrates of the species *Drosophila melanogaster* or *Caenorhabditis elegans*, and to their transgenic progeny. Preferably, the transgenic invertebrates contain the receptors according to the invention in a form which differs from that of the wild type.

10 The nucleic acids according to the invention can be prepared in the customary manner. For example, the nucleic acid molecules can be synthesized entirely chemically. In addition, only short segments of the sequences according to the invention can be synthesized chemically and these oligonucleotides can be labelled radioactively or with a
15 fluorescent dye. The labelled oligonucleotides can be used to screen cDNA libraries prepared from insect mRNA. Clones which hybridize to the labelled oligonucleotides ("positive clones") are selected for isolating the relevant DNA. After the isolated DNA has been characterized, the nucleic acids according to the invention are readily obtained.

20 The nucleic acids according to the invention can also be prepared by means of PCR methods using chemically synthesized oligonucleotides.

25 The nucleic acids according to the invention can be used for isolating and characterizing the regulatory regions which occur naturally adjacent to the coding region. Consequently, the present invention also relates to these regulatory regions.

30 The nucleic acids according to the invention can be used to identify novel active compounds for plant protection, such as compounds which, as modulators, in particular as agonists or antagonists, alter the conducting properties of the acetylcholine receptors according to the invention. For this, a recombinant DNA molecule, which encompasses

at least one nucleic acid according to the invention, is introduced into a suitable host cell. The host cell is cultured, in the presence of a compound or a sample which comprises a multiplicity of compounds, under conditions which permit expression of the receptors according to the invention. A change in the receptor properties can be detected, as described below in Example 2. Using this approach, it is possible to discover 5 insecticidal substances.

The nucleic acids according to the invention also make it possible to discover compounds which bind to the receptors according to the invention. These compounds can 10 likewise be used as insecticides on plants. For example, host cells which contain the nucleic acid sequences according to the invention and express the corresponding receptors or polypeptides, or the gene products themselves, are brought into contact with a compound or a mixture of compounds under conditions which permit the interaction of at least one compound with the host cells, receptors or the individual polypeptides.

15 Host cells or transgenic invertebrates which contain the nucleic acids according to the invention can also be used to discover substances which alter the expression of the receptors.

20 The above-described nucleic acids, vectors and regulatory regions according to the invention can additionally be used for discovering genes which encode polypeptides which are involved in the synthesis, in insects, of functionally similar acetylcholine receptors. According to the present invention, functionally similar receptors are understood as being receptors which encompass polypeptides which, while differing in their 25 amino acid sequences from the polypeptides described in this present publication, essentially possess the same functions.

Comments on the sequence listing and the figures:

SEQ ID NO: 1 shows the nucleotide sequence of the isolated Da7 cDNA, beginning
5 with position 1 and ending with position 2886. SEQ ID NO: 1 and SEQ ID NO: 2
also show the amino acid sequences of the protein deduced from the Da7 cDNA se-
quence.

SEQ ID NO: 3 shows the nucleotide sequence of the isolated Hva7-1 cDNA, begin-
10 ning with position 1 and ending with position 3700. SEQ ID NO: 3 and SEQ ID NO:
4 also show the amino acid sequences of the protein deduced from the Hva7-1 cDNA
sequence.

SEQ ID NO: 5 shows the nucleotide sequence of the isolated Hva7-2 cDNA, begin-
15 ning with position 1 and ending with position 3109. SEQ ID NO: 5 and SEQ ID NO:
6 also show the amino acid sequences of the protein deduced from the Hva7-2 cDNA
sequence.

Figure 1 shows the increase in intracellular calcium which occurs in cells which have
20 been recombinantly modified as described in Example 2 following the addition of
nicotine. Cells were loaded with Fura-2-acetoxymethyl ester (5 - 10 μ M in serum-
free minimal essential medium containing 1% bovine serum albumin and 5 mM cal-
cium chloride), washed with Tyrode solution buffered with N-(2-
25 hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (5 mM HEPES) and alternately
illuminated, under a fluorescence microscope (Nikon Diaphot) with light of 340 nm
and 380 nm wavelength. A measurement point corresponds to a pair of video images
at the two wavelengths (exposure time per image, 100 ms). The time interval be-
tween two measurement points is 3 s. After 8 images had been taken (measurement
30 point 4.0), nicotine was added to a final concentration of 500 μ M and the measure-
ment series was continued. The fluorescence intensity of the cells when illuminated

with light of 380 nm wavelength was divided by the corresponding intensity at 340 nm, thereby giving the ratio.

Examples:

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Example 1

Isolating the described polynucleotide sequences

10 Polynucleotides were manipulated using standard methods of recombinant DNA technology (Sambrook, et al., 1989). The bioinformatic processing of nucleotide and protein sequences was carried out using the GCG program package Version 9.1 (GCG Genetics Computer Group, Inc., Madison Wisconsin, USA).

15 Partial polynucleotide sequences

Sequence comparisons ("Clustalw") were used to identify regions, from which degenerate oligonucleotides were deduced by backtranslating the codons, of protein sequences from genes whose ability to form homooligomeric acetylcholine receptors 20 was known. In all, 5 such oligonucleotide pairs were selected for the polymerase chain reaction (PCR). Only one combination (see below) gave a product both from Heliothis cDNA and from Drosophila cDNA.

RNA was isolated from whole Heliothis virescens embryos (shortly before hatching) 25 using Trizol reagent (Gibco BRL, in accordance with the manufacturer's instructions). The same procedure was adopted with Drosophila embryos (24 h at 25°C). 10 µg of these RNAs were employed in a first cDNA strand synthesis (Superscript Pre-amplification System for first cDNA strand synthesis, Gibco BRL, in accordance with the manufacturer's instructions, reaction temperature 45°C).

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Subsequently, 1/100 of the abovementioned first-strand cDNA was in each case employed in a polymerase chain reaction (PCR) using the oligonucleotides alpha7-1s: (5'-GAYGTIGAYGARAARAAYCA-3') and alpha7-2a: (5'-
5 CYYTCRTCIGCRCRTTRTA-3') (recombinant Taq DNA polymerase, Gibco BRL). The PCR parameters were as follows: Hva7-1 and Hva7-2: 94°C, 2 min; 35 times (94°C, 45 s; 50°C, 30 s; 72°C, 60 s) and also Da7: 96°C, 2 min; 35 times (96°C, 45 s; 50°C, 30 s; 72°C, 60 s). In each case, this resulted in a detectable band of approx. 0.2 kb in an agarose gel (1%), both in the case of Drosophila cDNA and in the case of Heliothis cDNA. After the DNA fragments had been subcloned by means
10 of SrfScript (Stratagene), and their sequences had been determined, it turned out that two different DNA fragments had been amplified from Heliothis cDNA; these were 228-11 = Hva7-1 (partial, containing 165 bp) and 228-8 = Hva7-2 (partial, containing 171 bp). Only one DNA fragment was isolated from Drosophila cDNA; this was 248-5 = Da7 (partial, containing 150 bp).

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Isolating poly A-containing RNA from Heliothis virescens tissue and constructing the cDNA libraries

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The RNA for cDNA library I was isolated from whole Heliothis virescens embryos (shortly before hatching) using Trizol reagent (Gibco BRL, in accordance with the manufacturer's instructions). The RNA for cDNA library II was isolated from whole head ganglia from 500 Heliothis virescens larvae (stages 4-5) using Trizol reagent (Gibco BRL, in accordance with the manufacturer's instructions). The poly A-containing RNAs were then isolated from these RNAs by purifying with Dyna Beads
25 280 (Dynal). 5 µg of these poly A-containing RNAs were subsequently employed in constructing cDNA libraries I and II using the λ-ZAPExpress vector (cDNA Synthesis Kit, ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit, all from Stratagene). In a departure from the manufacturer's instructions, Super-script Reverse Transcriptase (Gibco BRL) was used for synthesizing the cDNA at a synthesis temperature of 45°C. In addition, radioactively labelled deoxynucleoside triphosphates were not added. Furthermore, the synthetized cDNAs were not frac-
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tionated through the gel filtration medium contained in the kit but instead through Size Sep 400 Spun Columns (Pharmacia).

Complete polynucleotide sequences

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Apart from the first screening round when isolating the Hva7-1 clone, all the screens were carried out using the DIG system (all reagents and consumables from Boehringer Mannheim, in accordance with the instructions in "The DIG System User's Guide for Filter Hybridization", Boehringer Mannheim). The DNA probes employed were prepared by means of PCR using digoxigenin-labelled dUTP. The hybridizations were carried out at 42°C overnight in DIG Easy Hyb (Boehringer Mannheim). Labelled DNA was detected on nylon membranes by means of chemiluminescence (CDP-Star, Boehringer Mannheim) using X-ray films (Hyperfilm MP, Amersham). Initial partial sequencing of the isolated gene library plasmids was carried out, for identification purposes, using T3 and T7 primers (ABI Prism Dye Terminator Cycle Sequencing Kit, ABI, using an ABI Prism 310 Genetic Analyzer). The complete polynucleotide sequences in Hva7-1, Hva7-2 and Da7 were determined, as a commissioned sequencing carried out by Qiagen, Hilden, by means of primer walking using cycle sequencing.

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a. Isolating the Da7 clone

10⁶ phages from a *Drosophila melanogaster* cDNA library in λ phages (Canton-S embryo, 2-14 hours, in Uni-ZAP XR vector, Stratagene) were screened using DIG-labelled 248-5 as the probe (in accordance with the manufacturer's (Stratagene) instructions). The maximum stringency when washing the filters was: 0.2 x SSC; 0.1% SDS; 42°C; 2 x 15 min. One clone (clone 432-1) was isolated whose insert had a size of 2940 bp (Da7, SEQ ID NO: 1). The largest open reading frame of this sequence begins at position 372 of the depicted sequence and ends at position 1822. The 770 amino acids polypeptide which is deduced from this (SEQ ID NO: 2) has a calculated molecular weight of 87.01 kD.

b. Isolating the Hva7-1 clone

10⁶ phages from the *Heliothis virescens* embryo cDNA library (library I) were included in the screening. The first of three screening rounds took place using α -³²P-labelled 228-11 DNA as the probe. The probe was hybridized to the filters in Quick-hyb (Stratagene) at 68°C for one hour. The filters were then washed twice, for 15 min on each occasion, at room temperature in 2 x SSC; 0.1% SDS and twice, for 30 min on each occasion, at 42°C in 0.1xSSC; 0.1% SDS. Hybridized probes were detected by means of autoradiography, at -80°C overnight, using XR X-ray films (Kodak) and employing intensifying screens (Amersham). The two further screening rounds were carried out using the DIG System (Boehringer Mannheim).

The clone 241-5, which was isolated in this screen, contained an insert of 3630 bp. This insert (Hva7-1, SEQ ID NO: 3) possesses a longest open reading frame which begins at position 335 of the depicted nucleic acid sequence and ends at position 1821. The 496 amino acids polypeptide which is deduced from this (SEQ ID NO: 4) has a calculated molecular weight of 56.36 kD.

c. Isolating the Hva7-2 clone

10⁶ phages from the *Heliothis virescens* ganglia cDNA library (library II) were included in the screening. Dig-labelled 228-8 DNA was used as the probe. The maximum stringency when washing the filters was: 0.1 x SSC; 0.1% SDS; 42°C; 2 x 15 min.

The clone 241-5, which was isolated in this screen, contained an insert of 3630 bp. This insert (Hva7-2, SEQ ID NO: 5) possesses a longest open reading frame which begins at position 95 of the depicted nucleic acid sequence and ends at position 1598. The 501 amino acids polypeptide which is deduced from this (SEQ ID NO: 6) has a calculated molecular weight of 56.71 kD.

Example 2

Generating the expression constructs

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a. Da7

The sequence region from position 372 to position 2681 of SEQ ID NO: 1 was amplified by means of a polymerase chain reaction (PCR). Deoxyoligonucleotides having the sequences GCGAATTCAACCACCATGAAAAATGCACAACTG and CGAGACAATAATATGTGGTGCCTCGAG were used for this. The Pfu polymerase from Stratagene was used as the DNA polymerase in accordance with the manufacturer's instructions. Following the amplification, the segment which had been generated was digested with the restriction endonucleases Eco RI and Xho I and cloned into a vector, i.e. pcDNA3.1/Zeo (Invitrogen), which had likewise been digested with Eco RI and Xho I.

b. Hva7-1

20 The sequence region from position 335 to position 1822 from SEQ ID NO: 3 was amplified by means of a polymerase chain reaction (PCR). Deoxyoligonucleotides having the sequences

GCAAGCTTACCACCATGGGAGGTAGAGCTAGACGCTCGCAC and GCCTCGAGCGACACCATGATGTGTGGCGC were used for this. The Pfu polymerase from Stratagene was used as the DNA polymerase in accordance with the manufacturer's instructions. Following amplification, the generated segment was digested with the restriction endonucleases HindIII and Xho I and cloned into a vector, i.e. pcDNA3.1/Zeo (Invitrogen), which had likewise been digested with HindIII and Xho I.

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c. Hva7-2

The sequence region from position 95 to position 1597 from SEQ ID NO: 5 was amplified by means of a polymerase chain reaction (PCR). Deoxyoligonucleotides
5 having the sequences GCAAGCGCCGCTATGGCCCCTATGTTG and TTGCACGATGATATGCGGTGCCTCGAGCG were used for this. The Pfu polymerase from Stratagene was used as the DNA polymerase in accordance with the manufacturer's instructions. Following amplification, the generated segment was digested with the restriction endonucleases HindIII and Xho I and cloned into a vector,
10 i.e. pcDNA3.1/Zeo (Invitrogen), which had likewise been digested with HindIII and Xho I.

d.Hva7-1 / 5HT₃ and Hva7-2 / 5HT₃ chimaeras

15 The region from position 335 to position 1036 from SEQ ID NO: 3 (Hva7-1/5HT₃ chimaera) and the region from position 95 to position 763 from SEQ ID NO: 5 (Hva7-2/5HT₃ chimaera) was in each case fused to the region from position 778 to position 1521 from the *Mus musculus* 5-HT₃ receptor cDNA (sequence in EMBL database: M774425) using the method of overlap extension (Jespersen et al. 1997).
20 The two fragments were subsequently cloned into the pcDNA3.1/Zeo vector by means of TA cloning (Invitrogen, in accordance with the manufacturer's instructions). Constructs containing the correct orientation of the two fragments in the vector were identified by sequencing using the T7 primer (Invitrogen).

25 Cell culture and gene transfer

HEK293 cells, which express the α subunit of an L-type Ca channel (Zong et al. 1995, Stetzer et al. 1996), were cultured in Dulbecco's modified Eagle's medium and 10% foetal calf serum at 5% CO₂ and from 20°C to 37°C. FuGENE 6 (Boehringer
30 Mannheim GmbH, Mannheim, Germany) was used for the gene transfer in accordance with the manufacturer's instructions. At from 24 h to 48 h after the gene trans-

fer, the cells were sown at various densities in microtitre plates. Recombinantly altered cells were selected by growth in Dulbecco's modified Eagle's medium and 10% foetal calf serum and 150 - 500 µg/ml of Zeocin/ml over a period of from 3 to 4 weeks. Individual resistant clones were analyzed as described below.

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Fura-2 measurements

The alterations in the intracellular calcium concentration were measured using Fura-2. A stock solution containing 2 mM Fura-2-acetoxy methyl ester (Sigma) in di-methyl sulphoxide (DMSO) was diluted to a final concentration of 5 - 10 µM in serum-free minimal essential medium (MEM, Gibco) containing 1% bovine serum albumin and 5 mM calcium chloride. The cells were incubated for from 45 to 60 min in this solution in a microtitre plate. The cells were then washed twice in Tyrode solution buffered with N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (5 mM HEPES) (HEPES-buffered salt solution containing 130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM NaHCO₃, 10 mM glucose). 100 µl Tyrode buffer were added to the wells of the microtitre plate and the cells were illuminated alternately, under a fluorescence microscope (Nikon Diaphot), with light of 340 nm and 380 nm wavelength. A series of video images (exposure time per image 100 ms) were taken with pauses of 3 seconds and stored, as digitalized images, in an image analysis computer (Leica, Quantimet 570). After 8 images had been taken (measurement point 4.0 in Fig. 1), nicotine was added to a final concentration of 500 µM and the measurement series was continued. The fluorescence intensity of the cells when illuminating with light of 380 nm wavelength was divided by the corresponding intensity at 340 nm and in this way a ratio was formed which represents the relative increase in calcium concentration (Grynkiewicz et al. 1985).

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